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EPA

Sperm Cell Test Using the Sea Urchin *Arbacia punctulata*

Supplemental Report for Training Videotape

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Supplemental Report for the

Sperm Cell Tests Using the Sea Urchin *Arbacia punctulata*

Training Videotape

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Sperm Cell Tests Using the Sea Urchin *Arbacia punctulata*

INTRODUCTION

The U.S. Environmental Protection Agency's (EPA's) Environmental Research Laboratory in Duluth, Minnesota, has developed a series of freshwater toxicity tests to evaluate effluent toxicity. The tests use freshwater fish, invertebrates, and plant species as indicators of toxic effects to lakes, streams, and rivers. The EPA Environmental Research Laboratory in Narragansett, Rhode Island (ERL-N), has adapted the freshwater toxicity testing approach and developed new methods to perform similar testing in the marine or estuarine environment.

This report summarizes methods developed at the Narragansett lab for estimating the chronic toxicity of marine or estuarine effluents and receiving waters on the gametes of the sea urchin, *Arbacia punctulata*. Sperm cells are exposed to effluents or receiving waters in a static system for 1 hour, then sea urchin eggs are added for 20 minutes of static exposure. The test measures the effect of exposure on fertilization. The methods described in this report and demonstrated in the accompanying tape are detailed in the EPA methods manual, "Short-term Tests for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms" (EPA/600/4-87/028).

OBTAINING AND MAINTAINING SEA URCHINS

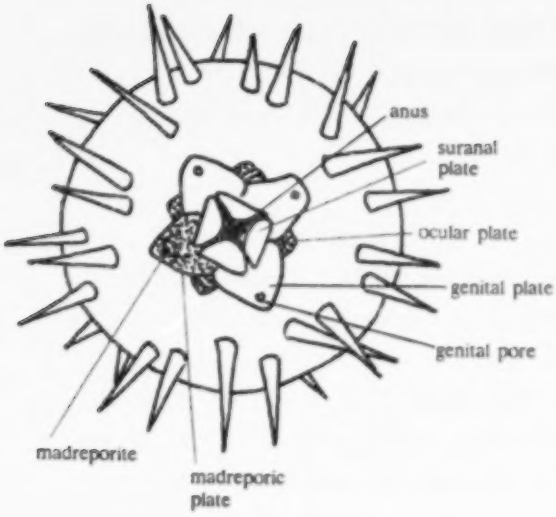
Adult sea urchins can be ordered from commercial biological supply houses, or collected along the Atlantic coast. Keep male and female animals in separate tanks; a 20-liter aerated tank can hold about 20 adults. Allow filtered seawater to flow into the tanks at a rate of 5 L/minute. The temperature should be 12° to 18° C.

Sea urchins are fed kelp of the species *Laminaria* gathered from Atlantic coastal waters or ordered from commercial supply houses. Supply the urchins with ample food, renewing the kelp each week and removing decaying kelp as necessary. Healthy sea urchins will attach to kelp or aquarium walls within hours - any unhealthy animals should be removed and should not be used for testing. Every 1 to 2 weeks, empty and clean the tanks.

A stock of at least 12 males and 12 females will be needed for routine testing. If the animals will be used on site, transport them separated by sex in separate or partitioned coolers packed with wet kelp and paper towels. On site, the sea urchins will be transferred into separate 10-gallon aquarium tanks with gravel-bed filtration. Even with filtration, be sure to change the water periodically to maintain good water quality.

OBTAINING GAMETES

Eggs and sperm are obtained from healthy animals collected from uncontaminated waters. Transfer the animals into a shallow bowl filled with enough seawater to just cover their shells. Four females should yield enough



Schematic of the aboral surface of Arbacia punctulata, with spines partly removed to show structure, especially the genital pores.

eggs to test 5 test dilutions plus 1 control, with 3 or 4 replicates. Eggs are obtained from the female sea urchins by electrical stimulation. Touch the shells close to the genital pores with electrodes from a 12-volt transformer for about 30 seconds. The red eggs will pool on the sea urchin shell above the genital pores. Collect the eggs from the shell using a 10-ml disposable syringe with a blunted large-gauge needle so that it will rest on the shell without puncturing it. After collection, remove the needle and empty the eggs into conical centrifuge tubes. All vials, pipets, and pipet tips used in this test should be soaked in seawater overnight. Keep the eggs at room temperature until use, but not longer than a few hours.

Obtain sperm from four male sea urchins. Again, place the animals in a shallow bowl with their shells just covered with control seawater. Like the females, the males are induced to spawn by placing electrodes from a 12-volt transformer against their shells for 30 seconds. The sperm appear white. Collect the concentrated sperm that pools on top of the shell using a 1-, 2-, or 3-ml syringe with a blunted large-gauge needle. Keep the sperm sample on ice, and record the collection time. The sperm are held on ice for 1 hour prior to exposure.

MAKING STOCK SOLUTIONS

In preparation for the actual exposure, the sperm and eggs are diluted to constant concentrations with control water. This ensures reproducibility in the test results. In Narragansett, laboratory workers use a standard data sheet for calculating and recording dilutions (sample data sheets are in Appendix D). Later, during the exposure period, 2,500 sperm will be present for every 1 egg.

After collection, the sperm should be in a volume of about 0.5 to 1 ml of control water in the collecting syringe. (Keep all sperm dilutions on ice.) This is called the "sperm stock" solution. Perform a 50% serial dilution for counting from this sample.

To do this, add 400 ul of the stock to 20 ml of control seawater in a vial labeled "A." Gently mix the solution by pipetting up and down with a 5 ml pipet, or by inverting the vial several times. The dilution in this vial is 1:50. Take 10 ml from vial A and add to 10 ml of water in the next vial, "B." After mixing solution B, take 10 ml out and add it to a third vial, "C," containing 10 ml of water. Vial C now contains a 1:200 dilution of the original sperm sample. Repeat the last step once more into a vial labeled "D." Discard 10 ml from vial D so that all vials contain 10 ml.

Next, make a 1:2,000 sperm suspension for counting. The sperm must be killed for counting, so add 10 ml of 10% glacial acetic acid in control water to vial C, the 1:200 dilution. Cap vial C, and mix by inversion. Add 1 ml from vial C to a vial labeled "E," which contains 4 ml of control water, and mix thoroughly. Vial E now contains 1/2,000th of the number of sperm in the original sperm sample. Now the concentration of the sperm stock can be determined. Place an aliquot of dilution E onto a hemacytometer for counting. Allow the dead sperm to settle for about 15 minutes.

Meanwhile, prepare a standard egg dilution of 2,000 eggs/ml. To wash the eggs, remove the supernatant water from the settled eggs. Add seawater and mix carefully by inversion. Spin the vial in a tabletop centrifuge at the lowest possible setting for 3 minutes to form a lightly packed pellet. Wash the eggs twice more. Discard the eggs and start again if the wash water appears red; this

means that the eggs are lysing and are unsuitable for testing.

After washing, transfer the washed eggs to a beaker containing 200 ml of control water. This is called the "egg test stock." Next, make a 1:10 dilution of the test stock for counting. Mix the stock solution using gentle aeration until the egg solution is homogeneous. The aeration device used in Narragansett is a 3-pronged diffuser attached by flexible tubing to an air pump. Cut the point from a pipet tip to make sure the eggs will not be damaged, then transfer 1 ml of egg solution to a vial containing 9 ml of control water. Mix by inversion.

Transfer 1 ml of the egg solution to a Sedgewick-Rafter counting chamber. Count the number of eggs under a dissecting microscope at 25X magnification. Ten times the number of eggs in that milliliter equals the number of eggs/ml in the egg stock. The target concentration is 2,000 eggs/ml. If less than 180 eggs were counted, allow the eggs to settle in the beaker, remove the proper volume of supernatant water according to the formula:

200 - (# of eggs counted) = ml to remove

If more than 200 eggs are counted, add the proper volume of water:

200 - (# of eggs counted) = ml to add

Verify the concentration by counting 1 ml of another 1:10 dilution of the adjusted stock solution. The count for the final dilution should equal 200 $\pm$ 20 eggs/ml. If there are four replicates, a test of five test concentrations with a control will require at least 25 ml of egg test stock solution at 2,000 eggs/ml.

When the sperm have settled in the hemacytometer, use a compound microsope to count the inner 400 squares on each side of the chamber. At 100X magnification, the sperm will appear as small specks in the chamber grid. Average the counts from each side of the hemacytometer. The resulting number times 10,000 is the number of sperm/ml in vial E. In turn, that number times 2,000 is the sperm concentration in the original sperm stock (because vial E contains a 1:2,000 dilution of the sperm stock solution). Calculate the sperm concentration in each dilution vial, then choose one that is greater than or equal to 5×10^7 sperm/ml. That vial is diluted to a final concentration of 50 million (5×10^7) sperm/ml and used as the sperm "test stock."

Only about 2.5 ml of sperm test stock solution are needed for testing 5 test solutions and a control, with 3 or 4 replicates. Hold the test stock on ice until the test begins, but no longer than 1 hour.

BEGINNING THE TEST

Store the effluent or receiving waters in a 4° C incubator or refrigerater until the tests begin, but not longer than 48 hours. Always maintain the salinity of the test samples at greater than 26 ppt. To do this, effluent samples may need to be adjusted using hypersaline brine. A recipe for hypersaline brine is provided in Appendix A. Receiving water samples with greater than 26 ppt salinity may be tested without further salinity adjustment. To test a series of decreasing concentrations of effluent, use a dilution factor of 0.5. When starting with effluent at 0 ppt salinity, ERL-N starts with 70 percent effluent. A table for preparing the samples is in Appendix A.

In Narragansett, disposable glass vials are used as test chambers. The vials are arranged in the partitioned cardboard box in which they are packaged. Perform a serial dilution from the highest concentration of effluent to the lowest, starting with 10 ml of the highest concentration. Move 5 ml from that vial to the next, and so on, finally discarding 5 ml from the last vial. There should also be at least 3 replicates of each concentration and the control solution. Warm the samples to 20° C by placing the vials on the laboratory bench if the ambient air is warm enough, or in an environmental chamber, before adding the sperm.

One hour after the sperm are collected, add 100 ul of the well-mixed sperm test stock to each test and control vial. Cover the chambers, record the time, and leave the chambers at room temperature for 1 hour.

Next, mix the egg test stock using gentle aeration and add 1 ml of that test stock solution to each exposure vial. When all of the vials contain eggs, lift the storage box and move it in circles to "swirl" and mix the egg-sperm suspension. Cover the chambers, record the time, and incubate the eggs and sperm at room temperature for 20 minutes.

After 20 minutes, end the test and preserve the samples by adding 2 ml of 10 percent formalin in seawater. Again, record the time, then cap the vials. It is best to evaluate the test immediately after the conclusion of the test. The vials may also be saved at room temperature until evaluation, but not longer than 24 hours.

EVALUATING THE TEST

This test demonstrates the effluent or receiving water's effect on sea urchin fertilization. To evaluate this, exposed and control eggs are examined under a microscope and the number of unfertilized eggs in each test chamber is recorded.

Cut the point from a pipet tip. For each replicate, transfer about 80 to 120 ul of the preserved eggs to a multiple-chamber counting slide. If a Sedgewick-Rafter counting chamber is used, transfer about 1 ml to the cell. Using a compound microscope at 100X magnification, count about 100 eggs. This should be done with adequate ventilation, preferably under a hood, because formalin is harmful when inhaled.

For each test chamber, record the total number of eggs counted, and the number that were not fertilized. Fertilized eggs are surrounded by a fertilization membrane, while unfertilized eggs lack this membrane. Abnormal eggs are not counted. For the test to be acceptable, the control chambers shou'd show more than 50 percent fertilization. The methods manual, "Short-term Tests for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms," explains how to analyze and interpret the results of the sea urchin test.

The sea urchin sperm cell test is currently used to assess the potential toxic effects of complex chemical mixtures on marine and estuarine organisms. Used in conjunction with chemical-specific methods, this test can provide a comprehensive and effective approach to assessing the impact of complex effluents discharged to the marine and estuarine environments.

REFERENCES

Aquatic Toxicity Testing Seminar Manual. 1985. U.S. EPA Environmental Research Laboratory, Narragansett, RI. ERL-N Contribution No. 796.

(Provides detailed descriptions of the methods used at Narragansett to evaluate the toxicity of discharges to marine and estuarine waters. Served as the basis of a series of seminars conducted by ERL-N personnel.)

Biomonitoring for Control of Toxicity in Effluent Discharges to the Marine Environment. 1989. U.S. EPA Center for Environmental Research Information, Cincinnati, OH; U.S. EPA Environmental Research Laboratory, Narragansett, RI. EPA/625/8-89/015.

(Describes the use of biomonitoring as an effective water quality-based approach to controlling the toxicity of discharges to estuarine and marine waters. Covers regulatory background, testing methods, and case studies.)

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. 1987. Environmental Monitoring and Support Laboratory, Cincinnati, OH. EPA/600/4-87/028.

(Describes methods, quality assurance, laboratory safety, facilities and equipment, data analysis, report preparation, and organism culture and handling for six short-term tests to estimate the chronic toxicity of effluents and receiving waters.)

Technical Support Document for Water Quality-based Toxics Control. 1985. U.S. EPA Office of Water Enforcement and Permits, Washington, D.C.

(Provides guidance for each step in the water quality-based toxics control process, from screening to compliance monitoring.)

APPENDIX A

PREPARING HYPERSALINE BRINE

BACKGROUND

Salinity adjustments are a vital part of using marine and estuarine species for toxicity testing. The majority of industrial and sewage treatment effluents entering marine and estuarine waters contain little or no measurable salts. Therefore, the salinity of these effluents must be adjusted before exposing estuarine or marine plants and animals to the solutions. In addition, it is important to maintain constant salinity across all treatments throughout the test for quality control. Finally, matching the test solutions' salinity to the expected receiving water's salinity may require salinity adjustments. ERL-N uses hypersaline brine, prepared from filtered natural seawater, to adjust exposure solution salinities. Note that commercially available artificial sea salts have not been sufficiently tested, and therefore are not recommended for all of the subchronic toxicity tests at this time.

Hypersaline brine has several advantages over artificial sea salts that make it more suitable for use in toxicity testing. Concentrated brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of test organisms. It may be held for prolonged periods without any apparent degradation. Brine may be added directly to the effluent to increase the salinity, or may be used as control water by diluting to the desired salinity with deionized water. The brine can be made from any high quality, filtered seawater supply through simple heating and aerating.

GENERATING THE BRINE

The ideal container for making brine from natural seawater has a high surface-to-volume ratio, is made of a non-corrosive material, and is easily cleaned. Shallow fiberglass tanks are ideal.

Collect high quality (and preferably high salinity) seawater on an incoming tide to minimize the possibility of contamination. Special care should be used to prevent any toxic materials from coming in contact with the seawater. The water should be filtered to at least 10 um before placing into the brine tank. Thoroughly clean the tank, aeration supply tube, heater, and any other materials that will be in direct contact with the brine before adding seawater to the tank. Use a good quality biodegradable detergent, followed by several thorough deionized-water rinses. Fill the tank with seawater, and slowly increase the temperature to 40° C. If a heater is immersed directly into the seawater, make sure that the heater components will not corrode or leach any substances that would contaminate the brine. A thermostatically controlled heat exchanger made from fiberglass works well.

Aeration prevents temperature stratification and increases the rate of evaporation. Use an oil-free air compressor to prevent contamination. Evaporate the water for several days, checking daily (or more or less often, depending on the volume being generated) to ensure that the salinity does not exceed 100 o/oo and the temperature does not exceed 40° C. If these changes are exceeded, irreversible changes in the brine's properties may occur. One such change noted in original studies at ERL-N was a reduction in the alkalinity of seawater made from brine with salinity greater than 100 o/oo, and a resulting reduction in the animals' general health. Additional seawater may be added to the brine to produce the volume of brine desired.

When the desired volume and salinity of brine is prepared, filter the brine through a 10-um filter and pump or pour it directly into portable containers (5-gallon Cubitainers or polycarbonate water cooler jugs are most suitable). Cap the containers, and record the measured salinity and the date the brine was generated. Store the brine in the dark at room temperature until used.

SALINITY ADJUSTMENTS USING HYPERSALINE BRINE

To calculate the volume of brine (V_b) to add to 0 o/oo sample to produce a solution at a certain salinity (S_f), use this equation:

$$V_b * S_b = S_f * V_f$$

where V_b = volume of brine, ml

S_b = salinity of brine, o/oo

S_f = final salinity, o/oo

V_f = final volume, ml (brine brought to this volume with 0 o/oo sample).

To calculate the volume of brine (V_b) required to raise the salinity of an effluent or receiving water sample (S_s) to a certain salinity (S_f), use this equation:

$$V_b = [(V_f * S_f) - (V_s * S_s)]/(S_b - S_f)$$

where V_b = volume of brine, ml

S_b = salinity of brine, o/oo

V_s = volume of sample, ml

S_s = salinity of sample, o/oo

S_f = final salinity , o/oo

V_f = final volume, ml (final volume is combined brine and deionized water plus the sample volume; percent original sample in the final sample = $V_s/V_f * 100$).

For the sea urchin test, start with an initial experimental concentration of 70 percent effluent (70 ml effluent at 0 o/oo + 30 ml brine at 100 o/oo), and perform a 0.5 serial dilution to make exposure concentrations of 35, 17.5, 8.8, and 4.4 percent effluent. The dilutions can be prepared directly in the test vials.

APPENDIX B

MATERIALS NEEDED FOR TESTING

Lab Supplies

Thermometer

Refractometer (salinometer)

Graduated cylinders, various sizes

1-liter volumetric flasks

Flat-bottomed glass dishes (about 20 cm diameter)

600- to 1000-ml glass beakers

Disposable gloves

Parafilm

Calibrated dispensing bottles (delivering 1 to 10 ml)

Pipettors: 5 ul, 50 ul, 200 ul, 1000 ul, 5 ml

Disposable pipet tips (200 ul and 1000 ul, with about 3 mm sliced off the point)

Ice bucket with cover

Conical centrifuge tubes appropriate for centrifuge

Test tube rack

1- to 3-ml and 10-ml syringes with blunt-tipped needles (cut off the point)

Aeration device for suspending egg solution

Disposable scintillation vials

Manual two-place cell counter

Sedgwick-Rafter counting chamber

Neubauer hemacytometer

Data sheets (Appendix D)

Pasteur pipettes with pipette bulbs

Multi-chambered counting slides

Lab Equipment

Incubator (capable of maintaining 20° C)

12-V transformer with steel electrodes

Centrifuge (capable of ambient, low-speed spin)

Vacuum pump, tubing, pasteur pipettes

Air pump (Silent Giant) or pressurized air source, with tubing

Fume hood

Dissecting microscope

Compound microscope

Solutions

Hypersaline brine

Deionized water

10% glacial acetic acid (reagent grade) in sea water

10% formalin in seawater

MATERIALS FOR MAINTAINING ORGANISMS

Sea urchins, *Arbacia punctulata* (about 1 dozen of each sex)

kelp, *Laminaria* sp.

Standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining sea water at 15° C) with appropriate filtration and aeration system

APPENDIX C

SUMMARY OF TEST CONDITIONS

Test type:	static, non-renewal
Salinity:	30 o/oo $\pm$ 2o/oo
Temperature:	19 to 21 °C
Light source:	ambient laboratory light
Irradiance:	about 10-20 uE m^{-2} s^{-1}
Test solution volume:	5 ml
Test chamber size:	20-ml disposable, glass liquid scintillation vials, not pre-cleaned
Number of treaments per test:	minimum of 5 effluent concentrations and one control
Number of test organisms:	pooled eggs from 4 females and pooled sperm from 4 males per test
Number of eggs and sperm cells per treatment:	about 2,000 eggs and 5,000,000 sperm per vial
Number of replicate chambers per treatment:	4 (minimum of 3)
Dilution water:	uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts
Dilution factor:	0.5
Test duration:	1 hour and 20 minutes
Effect measured:	fertilization of sea urchin eggs

APPENDIX D
DATA SHEETS

SPERM CELL TOXICITY TEST
DATA SHEET # 1

TESTID:
PERFORMED BY:

SPERM SOLUTIONS

HEMACYTOMETER CT, E: x 10^4 = spm 'E'
sperm concentrations: E x 40 = A = SPM
E x 20 = B = SPM
E x 5 = D = SPM

SOLUTION SELECTED FOR TEST (>= 5 x 10^7 SPM):
DILUTION: SPM/(5 x 10^7) = DF
((DF) x 10) - 10 = + SW, ML

EGG SOLUTIONS

INITIAL COUNT: = VOLUME FOR FINAL EGG STOCK

COUNT ON FINAL EGG STOCK: (200 x 10 EGGS/ML)

TEST STOCKS
SPERM STOCK: (5 x 10^7 SPM)
VOLUME ADDED/TEST VIAL: (100 UL)

EGG STOCK: (2000/ML)
VOLUME ADDED/TEST VIAL (1 ML)

TEST TIMES

SPERM COLLECTION: _____

SPERM ADDED: _____

EGGS ADDED: _____

FIXITIVE ADDED: _____

SAMPLES READ: _____

SALINITIES:

SPERM CELL TOXICITY TEST RAW DATA

VERIFICATION:

TESTID: STUDY: PROJECT:

SCONTROL1: SCONTROL2: SCONTROL3:

EFF1: EFFCTL1:

EFF2: EFFCTL2:

EFF3: EFFCTL3:

TESTCOM:

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
General Comments:				3	100	

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
General Comments:				3	100	

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
General Comments:				3	100	

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
General Comments:				3	100	

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